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THE ROLE OF LH PULSES ON *IN VITRO* FOLLICULAR DEVELOPMENT IN THE METESTROUS RAT OVARY

by

SHIRLEY JANE LUTTMER

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE

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VITA

The author, Shirley Jane Luttmer, is the daughter of William and Thea Luttmer. She was born on July 31, 1959 in Libertyville, Illinois.

She obtained her primary education at Santa Maria del Popolo Elementary School in Mundelein, and secondary education at Carmel High School for Girls in Mundelein, graduating in May, 1977.

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CHAPTER I

REVIEW OF LITERATURE

Ovarian Anatomy

The ovary is a highly complex organ which contains interstitial cells, follicles and corpora lutea. The smallest follicle within the ovary is the primordial follicle which is formed when a primary oocyte becomes surrounded by a single layer of relatively undifferentiated, flattened stromal cells (Pedersen, 1970; Peters, 1979). These primordial follicles represent the pool of non-growing follicles from which developing follicles emerge. When a primordial follicle starts to grow, its oocyte begins to enlarge and the single layer of follicular cells divides, forming a stratified layer of granulosa cells which surrounds the primary oocyte (Pedersen, 1972). Granulosa cells are polyhedral and their cytoplasm contains evenly distributed ribosomes, oblong mitochondria with lamellar cristae, vesicles and cisternae of smooth and rough endoplasmic reticulum and golgi complexes (Guraya, 1973; Peluso *et al.*, 1980a; Motta and Sayoko, 1981). The oocyte grows and reaches its maximum size when it is covered by four layers of granulosa cells and before an antrum appears (Mandl and Zuckerman, 1952). Once the initiation of follicular proliferation occurs, the follicle will either mature and ovulate or become atretic

(Pedersen, 1970). Atresia can occur at any time in follicular development (Byskov, 1974).

Theca cells differentiate from the interstitial cells and surround the granulosa cell layers, but remain separated from them by a basement membrane. These theca cells differentiate into the highly vascular secretory theca interna and the outer connective tissue, the theca externa. The steroid-secreting theca interna cells contain spherical mitochondria with tubular or vesicular cristae, endoplasmic reticulum, and lipid droplets (Guraya, 1973; Peluso et al., 1980a; Motta and Sayoko, 1981).

As the follicle begins to expand, the follicular cells secrete a fluid which accumulates into one large pool forming a cavity or antrum located in the center of the follicle (Pedersen and Peters, 1968). Both granulosa and theca cells contribute to the formation of follicular fluid which contains various proteolytic enzymes, proteins and steroids (Edwards, 1974). Once the antrum appears, the follicle is known as an antral follicle. Ovulation and luteinization are the terminal stages of differentiation for large, non-atretic follicles (Richards and Midgley, 1976). Ovulation occurs when the follicle ruptures and the ovum is extruded, leaving behind the follicle shell which then differentiates into a corpus luteum (Greenwald, 1974).

Ovarian Changes During The Estrous Cycle

The anatomy of the ovary changes with each day of the reproductive cycle. These changes have been described in detail for the laboratory rodents, the mouse and rat which possess four-day estrous cycles. Ovulation occurs shortly after midnight on estrus (Day 1). Any large follicles that did not ovulate on estrus undergo atresia. Corpora lutea form and begin to secrete progesterone. Also during estrus, the rate at which a cohort of medium-sized (250-350 um in diameter) follicles is growing increases. Throughout metestrus (Day 2) this cohort of follicles continues to grow. The corpora lutea regress on diestrus (Day 3) and follicles continue to develop. By proestrus (Day 4) many large preovulatory-size follicles (>550 um in diameter) have developed. These follicles are now capable of ovulating in response to the preovulatory gonadotropin surge which occurs during the evening of proestrus, thus completing a four-day cycle.

Kinetics of Follicular Growth

The initiation of follicular growth occurs continuously during each day of the estrous cycle (Peters et al., 1975). Thus, different-sized follicles are found within the ovary on any day of the cycle. The rate at which follicles grow appears to be related to both the size of the follicle as well as the day of the cycle. Primordial follicles represent the pool of non-growing follicles. The preantral

follicles ($\leq$ 250 um in diameter) are growing, whereas medium (250-350 um in diameter), large (350-550 um in diameter), and preovulatory (> 550 um in diameter) follicles are either growing or undergoing atresia.

The rate at which follicles grow can be deduced by determining the percentage of the granulosa cell population that is synthesizing DNA. By examining autoradiographs prepared after pulse-labelling with tritiated thymidine, the number of cells synthesizing DNA can be determined and expressed as a percentage of the total number of cells within a follicle (labelling index).

Pulse-labelling studies of mature mouse (Pedersen, 1970; Pedersen, 1972) and rat (Hirshfield and Midgley, 1978a; Groen-Klevant, 1981) ovaries reveal that throughout the cycle, the number of growing preantral follicles is relatively constant; thus, the same number enter this group as leave. Medium antral follicles grow fastest at estrus. This is reflected in the values for the labelling index (the percentage of labelled cells within a cell population; in this case, the follicle), length of the S-phase (time in which the cells are in the DNA-synthesis phase) and doubling time (the time it takes the granulosa cells to double their number) of the follicle. More specifically, in small follicles that are beginning to form antra, the labelling index is 15% in the mouse (Pedersen, 1970; Pedersen and Peters, 1971; Pedersen, 1972) and 11% in the rat (Groen-Klevant, 1981). In medium antral follicles, however, the labelling

index is 32% in the mouse (Pedersen, 1970; Pedersen and Peters, 1971; Pedersen, 1972) and 43% in the rat (Peluso and Downey, 1982). Thus, the labelling index increases with an increase in follicle size (Hirshfield and Midgley, 1978a). To determine the doubling time of the follicle, the duration of the S-phase is needed. In the mouse, the S-phase shortens from ten hours in medium-sized follicles to seven hours in large-sized follicles (Pedersen, 1970; Pedersen and Peters, 1971; Pedersen, 1972). Furthermore, the doubling time shortens with an increase in follicle size (Pedersen, 1970). Thus, during follicular development, more granulosa cells proliferate and the mitotic cycle is shortened. Therefore, the increase in the labelling index and the decrease in the S-phase and doubling time accounts for the increased number of large follicles on late estrus and metestrus.

Hormonal Control of Follicular Growth

Follicular growth is a complex process which is controlled by numerous regulatory agents. The initiation of follicular growth, however, appears to be independent of gonadotropin stimulation (Peters et al., 1975; Nakano et al., 1975). Consequently, follicles with a diameter of less than 250 um grow at a constant rate and the number of these follicles does not change significantly during the estrous cycle (Pedersen, 1972). The continued growth of medium-sized and large-sized follicles, on the other hand, is

dependent upon hormonal stimulation (Peters et al., 1975; Moore and Greenwald, 1974).

Preantral follicular growth is dependent on a complex interaction between the following hormones: Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), progesterone, estrogen and androgen. FSH increases the rate at which small follicles grow (Greenwald, 1973). The growth of these follicles results from granulosa cell divisions (Pedersen, 1972). When a follicle is stimulated by FSH, DNA synthesis is rapidly initiated with further mitoses being dependent on further FSH stimulation (Ryle, 1969a; Ryle, 1969b; Ryle, 1972; Lunenfeld et al., 1975; Eshkol et al., 1969).

FSH stimulates progesterone and estrogen secretion in the granulosa cells of the preantral follicle (Nuti et al., 1974; Hillier et al., 1978), via a membrane associated FSH-sensitive adenylate cyclase system (Hillier et al., 1978). FSH stimulates progesterone secretion by binding to the FSH receptor, inducing 3',5'-cAMP which in turn stimulates the conversion of cholesterol into pregnenolone (Hillier et al., 1978). Androgens enhance both the FSH-induced progesterone and estradiol secretion both in the rat (Armstrong and Dorrington, 1976) and in the pig (Schomberg et al., 1978; Schomberg, 1979). Progesterone stimulates the growth of preantral follicles (Kent, 1973). Estrogen also promotes granulosa cell divisions (Goldenberg et al., 1972a; Goldenberg et al., 1972b). Granulosa cells have specific cytosolic and nuclear receptors for estrogen thus, estrogen can

exert its effects directly upon granulosa cells (Richards and Midgley, 1976). Estradiol administered to immature hypophysectomized rats not only stimulates granulosa cell proliferation within preantral follicles (Goldenberg et al., 1972b; Ireland and Richards, 1978a), but also increases the number of estrogen receptors (Richards et al., 1979; Rani et al., 1981). Estrogen also induces the capacity of FSH to maintain and increase its own receptors within the granulosa cells of the preantral follicle (Ireland and Richards, 1978a). In the presence of estrogen, FSH also can induce antrum formation within the preantral follicle (Goldenberg et al., 1972a; Goldenberg et al., 1972b). Thus FSH stimulates the growth of preantral follicles by promoting both progesterone and estrogen secretion. Both of these steroids induce granulosa cell divisions and thereby growth of the preantral follicles.

Once an antrum begins to develop, LH acts synergistically with FSH for the enrichment of the vascular development of the antrum, and stimulation of steroid enzyme activity within the thecal and granulosa cell layers (Kraiem and Samuels, 1974). FSH also induces the acquisition of specific LH receptors within the granulosa cells (Richards and Midgley, 1976; Hillier et al., 1978; Erickson et al., 1979). Estrogen stimulates this process within the developing antral follicles (Richards et al., 1976; Richards and Midgley, 1976; Sanders and Midgley, 1982). LH acts synergistically with FSH to induce LH receptors within granulosa cells

(Ireland and Richards, 1978b). These LH receptors within the antral follicle appear to be limited to the mural layers of granulosa cells (Jonassen and Richards, 1980). LH simulates the growth of antral follicles (Ryle, 1969c). LH acts upon the thecal cells of the preantral follicle by binding to specific LH receptors on the thecal cell plasma membrane (Zeleznik et al., 1974), enhancing thecal cell divisions (Ryle, 1972), and stimulating the production of aromatizable androgens within the thecal cells (Armstrong and Papkoff, 1976; Fortune and Armstrong, 1977; Richards and Midgley, 1976; Carson et al., 1981b). The androgen is then aromatized into estrogen in the granulosa cells under the influence of FSH (Dorrington et al., 1975; Moon et al., 1975; Dorrington, 1977). The ability of follicles to synthesize estrogen is dependent upon thecal androgen production and the presence of aromatizing enzymes within the granulosa cells (Richards et al., 1979). Since granulosa cells are the exclusive site for FSH receptors in the ovary (Zeleznik et al., 1974), FSH can act directly on granulosa cells to induce aromatizing enzymes and stimulate estrogen synthesis (Dorrington et al., 1975; Moon et al., 1975; Armstrong and Dorrington, 1976; Erickson and Hsueh, 1978). In summary, specific changes that occur in antral follicles in the presence of LH and FSH are associated with increases in: 1) follicular steroidogenesis including increased estrogen production (Richards et al., 1980; Bogovich et al., 1981) and increased thecal androgen biosynthesis (Richards et al.,

1980; Bogovich et al. 1981); 2) LH receptor content for both thecal and granulosa cells (Richards and Kersey, 1979); and 3) hormone responsive adenylate cyclase activity (Richards and Kersey, 1979). Once the small antral follicles possess aromatase activity and LH receptors, LH as well as FSH can increase aromatase activity (Bogovich et al., 1981). This process is critical in the growth of the follicle because with their steroidogenic capacity, follicles can now respond to the LH surge on proestrus and luteinize.

The degree to which granulosa cells of preantral follicles are exposed to FSH may be a major determinant of the follicular response to androgens of thecal origin. Follicles from estrogen-primed hypophysectomized immature rats treated with appropriate amounts of FSH exhibit increased sensitivity to androgen-responsive steroidogenesis whereas treatment with a "deficient" amount of FSH results in atresia in response to androgen (Hillier et al., 1980). Treatment with FSH can rescue follicles in an early stage of atresia (Hirshfield and Midgley, 1978b; Byskov, 1979) by maintaining granulosa cell division (Peluso and Steger, 1978). Atretic follicles have reduced aromatase activity (Carson et al., 1981a). Thus, the decrease in the aromatizing enzymes leads to an increase in the concentration of testosterone within the follicle. Granulosa cells have specific receptors for testosterone (Schreiber et al., 1976), thus the increase in testosterone may act directly upon the granulosa cells to induce atresia. In hypophysectomized

immature rats, androgens produced in response to LH act locally to inhibit the effects of estrogen on follicular growth (Louvet et al., 1975). Only in non-atretic follicles are androgens capable of being converted to estrogen, thereby avoiding the harmful effects of excess androgen on the growing follicle and therefore atresia. This accounts for the conflicting reports that androgens both stimulate follicular growth (Beyer et al., 1974; Kumari et al., 1978) and promote follicular atresia (Louvet et al., 1975; Harmen et al., 1975).

Development of Preovulatory Follicles During the Estrous Cycle

The secondary surge of FSH which occurs on the morning of estrus recruits follicles destined to develop into preovulatory follicles for the next cycle (Hirshfield, 1979). When serum FSH concentrations are abruptly lowered on the morning of estrus, rapid onset of atresia occurs in large antral follicles (Uilenbroek et al., 1976). In addition, reduction of the secondary FSH surge by injection of FSH antiserum at proestrus delays preovulatory follicle development (Welschen and Dullart, 1976; Schwartz et al., 1973). More specifically, inhibition of the primary FSH surge does not block follicular recruitment whereas inhibiting the secondary FSH surge by porcine follicular fluid prevents the recruitment of small antral follicles (Hoak and Schwartz, 1980). Thus, the secondary surge of FSH is essential for the recruitment of the next crop of follicles for the next

ovulation (Welschen, 1973; Schwartz, 1974; Hirshfield and Midgley, 1978b; Hoak and Schwartz, 1980).

Once follicles are recruited, LH stimulates thecal cell androgen production, and estradiol and LH receptors within the granulosa cells of the follicle (Bogovich et al., 1981). Although FSH is involved in the maturation of small antral follicles at the beginning of a new cycle, it may actually be the subtle increases in LH that are required for preovulatory follicular development (Bogovich et al., 1981). These subtle but sustained increases in basal LH are required to initiate thecal androgen production (Bogovich et al., 1981), follicular estrogen production and LH receptor induction between diestrus and proestrus (Richards et al., 1980). Therefore, the sustained increases in LH activity can stimulate the growth of small antral follicles to preovulatory follicles (Richards and Bogovich, 1982) in the presence of serum progesterone (Bogovich et al., 1981).

In the mature follicle, LH induces specific receptors for prolactin on granulosa cells (Richards and Williams, 1976; Rao et al. 1977). FSH also stimulates the formation of prolactin receptors in granulosa cells (Richards and Midgley, 1976; Wang et al., 1981). The highest binding activity for prolactin occurs during proestrus (Cheng, 1976). The function of prolactin receptors on granulosa cells is unclear but in antral follicles, estrogen acts synergistically with prolactin to produce progesterone (Smith, 1980; Veldhuis et al., 1981).

CHAPTER II

STATEMENT OF THE PROBLEM

Although basal LH levels are low on metestrus and diestrus (Butcher *et al.*, 1974), these low levels of LH appear to be necessary for continued follicular growth and estrogen synthesis (Richards and Bogovich, 1982). LH is secreted in a pulsatile manner (Gallo, 1981) with LH pulses ranging from 20 ng/ml to 60 ng/ml and occur at circhoral intervals (Gallo, 1981). The fact that during follicular growth LH is released in a pulsatile manner rather than continuously may be important since tonic hormonal secretion to target tissue often results in a loss of responsiveness to that tonic input, while pulsatile secretion does not result in a loss of responsiveness (Catt *et al.*, 1979; Hunzicker-Dunn *et al.*, 1978; Hunzicker-Dunn and Birnbaumer, 1981; Powell *et al.*, 1981).

Many *in vitro* culture systems have been developed to examine follicular growth and development in response to physiological stimuli. With these culture systems, however, the precise level of hormonal input is not possible to determine unless a tonic hormonal stimulus is used. Therefore, Peluso and Gruenberg (1983) developed an *in vitro* culture system in which the effects of pulsatile physiological stimuli upon a tissue sample, can be assessed. Utilizing

this culture system in which physiological pulses of LH can be administered to ovaries and the precise concentration of the hormone determined at any time during the culture period, it has been shown that exposing ovaries to circhoral pulses of LH which mimic those on metestrus, results in follicular growth patterns that resemble those of the intact cycling rat (Peluso *et al.*, 1983). It is hypothesized that the amplitude of the LH pulse wave may play an important role in stimulating follicular growth. Therefore, experiments were designed to determine the effect of the amplitude of LH pulses on *in vitro* follicular growth in the metestrous rat ovary.

CHAPTER III

MATERIALS AND METHODS

Preparation of Media

Bovine Luteinizing Hormone (NIAMDD-bLH-10) with a biopotency of 1.06 NIH-LH-S1 units/mg (one unit of activity = 1 mg of NIH-LH-S1) and bovine Follicle-Stimulating Hormone (NIAMDD-bFSH-1) with a biopotency of 0.49 NIH-FSH-S1) were used in this experiment. Stock solutions of LH (10 mg NIH-LH-B10 dissolved in 50 ml of Medium-199 for a concentration of 200,000 ng NIH-LH-B10/ml) and FSH (10 mg NIH-FSH-B1 dissolved in 50 ml of Medium-199 for a concentration of 200,000 ng NIH-FSH-B1/ml) were mixed separately and frozen at -200C until added to culture media. LH and FSH concentrations of the solutions were expressed in RP-1 units.

Gonadotropins were added to Medium-199 which was supplemented with 2.2 g/L $NaHCO_2$, 2 g/L Bovine Serum Albumin, 50 mg/L Streptomycin Sulfate, 62.9 mg/L K-Penicillin-G, and 2.38 g/L Hepes (Gibco, Detroit, Michigan). All media were subsequently equilibrated with 5% CO_2 in oxygen and the pH adjusted to 7.4. The media were then transferred to holding flasks or culture chambers and stored at 5°C for up to 4 days. All bottles were warmed to 25°C, injected with 200 I.U./L regular insulin (Lente) and equilibrated with 5% CO_2 in oxygen 20 min. prior to use. During the experiment,

media in the holding flasks were gassed for 1 min. in every 10 min. interval with 5% CO_2 and oxygen. Gas was allowed to enter the holding flask by opening an in-line solenoid valve (ITT-General Controls, Glendale, Ca.; Model S301). The solenoid valve was controlled by a Chrontrol microprocessor controller (Lindberg Enterprises, San Diego, Ca.).

Experimental Protocol

Female Charles River Wistar rats (5 to 6 mos. old) were housed under controlled conditions of humidity (50%), temperature (24° C) and photoperiod (12h light/24h). The lights were turned on at 600h. The animals were fed Purina Lab Chow and water *ad libitum*. Estrous cycles were monitored by vaginal smears taken daily between 0800 and 1000h. Animals with at least three consecutive 4 or 5-day estrous cycles were used for this experiment. As assessed by vaginal smear cytology, a rat was considered to be in metestrous if its vaginal smear was leukocytic and preceded by a predominantly cornified vaginal smear. Only healthy-appearing rats without signs of respiratory distress, mammary tumors, or other gross pathologies were used. For this experiment, all metestrous rats were autopsied between 1000 and 1015h except the four *in vitro* control rats two of which were sacrificed at 1030h and the other two after a 3h period at 1330h.

At autopsy the ovaries were removed, trimmed of fat and placed im Medium-199 which was supplemented with 400

I.U./L heparin sulfate and 200 I.U./L insulin. The ovaries were incubated at room temperature in heparinized-media for 2 to 4 minutes and then placed in a separate culture chamber to which 10 ml of Medium-199 was added.

In this study, metestrous ovaries were exposed to the following hormonal treatments: 1) no gonadotropic stimuli (in vitro control); 2) tonic FSH (200 ng FSH-RP-1/ml); 3) tonic LH (30 ng LH-RP-1/ml); 4) tonic FSH and tonic LH; 5) tonic FSH and pulsatile LH (20ng-40ng-20ng); and 6) tonic FSH and pulsatile LH (10ng-50ng-10ng). A comparison of the tonic LH, tonic FSH and pulsatile LH (amplitude = 40 ng/ml), and tonic FSH and pulsatile LH (amplitude = 50 ng/ml) treatment groups is illustrated in Figure 1. Regardless of LH treatment, the total amount of LH exposed to each ovary was 3060 ng over the 3h culture period.

In order to induce LH pulses, two peristaltic pumps were connected to the culture chamber (a 20 ml syringe) which was housed in a 37°C incubator (Fig. 2). A chrontrol microprocessor controller (Lindberg Enterprises, San Diego, Ca.) controlled the pumps in such a way that the first pump delivered LH concentrating media to the culture chamber for the first half hour, thereby increasing LH levels within the culture chamber (Table 1). Simultaneously, the microprocessor controller turned off the LH concentrating pump and switched on the LH diluting pump for the remaining 30 minutes of the hour, thus decreasing the concentration of LH within the culture chamber. Both pumps delivered media at a

rate of 34 ml/h. This schedule was repeated for a 3h time period.

Culture System

The culture system and associated mathematical considerations have been previously published (Gruenberg and Peluso, 1983). Basically, the concentration of LH within the culture chamber at any point in time was calculated using the following equation:

$$C(t) = C_f + (C_i - C_f)\, e^{\frac{-RT}{V}}$$

where:

$C(t)$ = concentration in culture chamber at time (t)

C_f = concentration in holding flask (final culture concentration at $t = \infty$)

C_i = initial concentration within culture chamber ($t = 0$)

R = media delivery from holding flask to culture chamber (ml/h)

T = time in hours

V = volume of media in culture chamber (ml)

Analysis of Follicular Development

After culture, the ovaries were fixed in Bouin's solution, dehydrated, embedded in paraffin, and sectioned at 10 um. Histological examinations were made on follicles 160 um in diameter or greater. Largest diameters were determined

by measuring follicles containing the oocyte with the nucleus and/or nucleolus visible (Peluso and Downey, 1982). The follicles were classified as atretic if 3 or more pyknotic nuclei were observed within the granulosa cell layers or antrum in the largest section (Peluso and Downey, 1982). A Zeiss Videoplan Image Analyzer was used to determine the total number of granulosa cells for each follicle. The volume of the entire follicle, the antral cavity and the ovum were calculated by measuring their perimeters in the largest section of the follicle. By using these perimeter measurements, the volume of each follicular component was mathematically determined using the Zeiss Videoplan software. The volume of the antral cavity and ovum was subtracted from the volume of the entire follicle to determine the volume of the granulosa cell layer. To calculate the total number of granulosa cells in a follicle, the volume of the granulosa cell layer was divided by the average volume of a granulosa cell. The average granulosa cell volume used to calculate the total number of cells per follicle was 100 um^3. The average cell density of the granulosa cells was 9.3 cells/625 um^2. Neither the cell volume nor the granulosa cell density was affected by any of the *in vitro* treatments (Table 2).

To assess the net increase in the total number of cells per follicle in response to various treatments, the total number of cells from the largest follicle in the controls was subtracted from the total number of cells in the

largest follicle of each treatment group. Pairing was continued until all the 160-320 x 10^3 cell-size follicles in each treatment group were accounted for. The data was expressed as both the net increase and as the % increase in the total number of cells per follicle.

Statistical comparisons between and within the different hormone treatment groups were evaluated using either chi-square analysis or a paired t test.

CHAPTER IV

RESULTS

Between 1030h and 1330h, the % normal follicles with 80-159 x 10^3 cells increased. This reflects a decrease in the rate of atresia, since the total number of follicles in this size class was not increased. In addition, both the total number of follicles and % normal follicles in the 160-320 x 10^3 cell-size class increased (Fig. 3). This growth of follicles into the 160-320 x 10^3 cell-size range was due to an average increase of 55,542 cells (35.8%) per follicle (Fig. 9). When the 1030h *in vivo* controls were compared with the *in vitro* controls, no change in follicular distributions or % normal follicles within each size class was observed (compare upper panel of Fig. 3 with upper panel of Fig. 4).

FSH treatment decreased the total number of follicles with 80-159 x 10^3 cells by 10% ($p<0.05$) while increasing by 11% the number of follicles within the 10-39 x 10^3 cell-size class (Fig. 4). Ovaries incubated with tonic LH also showed a decrease in the total number of follicles with 80-159 x 10^3 cells ($p<0.05$), and an increase in the total number of follicles with 160-320 x 10^3 cells ($p<0.05$). Tonic LH also increased the % normal follicles in both cell-size classes (Fig. 5). This growth of follicles into the 160-320 x10^3

cell-size range was due to an average increase of 55,868 cells (35%) per follicle (Fig. 9).

When ovaries were incubated with both tonic FSH and tonic LH, the total number of follicles with 80-159 x 10^3 cells decreased by 9% ($p<0.05$), while the total number of follicles from 10-39 x 10^3 cells increased by 11% (Fig. 6). Follicular development within ovaries incubated with FSH and pulsed LH (amplitude = 40ng/ml) were similar to the *in vitro* controls except that the % normal follicles within the 20-39 x 10^3 cell-size class increased ($p<0.05$; Fig.7). However, ovaries incubated in FSH and LH pulsed with amplitudes of 50 ng/ml showed a decrease of 5% in the total number of follicles from 80-159 x 10^3 cells and an increase of 6% ($p<0.05$) in the total number of follicles from 160-320 x 10^3 cells (Fig. 8). This growth of follicles into the 160-320 x 10^3 cell-size range was due to an average increase of 26,720 cells (17.5%) per follicle (Fig. 9). A summary of the effects of the various hormonal treatments on follicular growth and atresia in the metestrous rat ovary is shown in Figure 10.

CHAPTER V

DISCUSSION

This study indicates that LH plays a dominant role in stimulating the growth of mid-sized antral follicles in cultured metestrous rat ovaries. Low levels of LH can stimulate mid-sized antral follicular growth in intact, progesterone implanted prepubertal rats (Richards and Bogovich, 1982; Richards et al., 1980). During metestrus (Butcher et al., 1974; Gallo, 1981) and late pregnancy (Richards, 1979; Richards and Kersey, 1979; Bogovich et al., 1981), LH levels are low and serum progesterone levels are elevated. In both of these physiological states, LH potentiates the growth of mid-sized antral follicles with progesterone facilitating the actions of LH (Richards and Bogovich, 1982). Although LH levels are low, LH is secreted in discrete hourly pulses in metestrous rats. Hourly pulses of LH (60 ng/ml in amplitude) prevent the atresia of mid-sized antral follicles, increase the percent non-atretic and the percent growing mid-sized antral follicles (Peluso et al., 1983).

LH induces these changes within ovarian follicles by stimulating thecal cell differentiation (Ryle, 1972). Thecal cells produce androgen in response to LH (Armstrong and Papkoff, 1976; Fortune and Armstrong, 1977; Richards and Midgley, 1976; Carson et al., 1981b). This androgen is then

aromatized into estrogen within the granulosa cells (Dorrington et al., 1975; Moon et al., 1975; Dorrington, 1977). Estrogen in turn promotes granulosa cell divisions (Goldenberg et al., 1972a; Goldenberg et al., 1972b). By this mechanism, LH exerts its actions upon the follicle to induce follicular growth.

Follicular growth due to the administration of tonic LH in vitro results in a 35% increase in the number of granulosa cells per follicle. Hourly LH pulses of 50 ng/ml in amplitude also stimulate mid-sized antral follicular growth and this growth is due to a 17.5% increase in the number of cells per follicle. Assuming that follicular growth occurs exponentially, the apparent cell cycle length (t_{Ca}) or cell doubling time (t_D) can be calculated from the following equation:

$$t_D = t_{Ca} = \frac{\ln 2}{K_G}$$

where k_G = the fraction of new cells generated per hour (Aherne et al., 1977). Thus, the appropriate calculation reveals an apparent cell cycle length of 5.8 h for in vivo controls, 5.9 h for tonic LH treatment and 11.9 h for treatment with FSH and LH pulses of 50 ng/ml in amplitude. In the mouse the apparent cell cycle length is 17 h (Pedersen, 1970). However, the labelling index of mid-sized antral follicles of the metestrous mouse (32%) (Pedersen, 1972) is

1/4 lower than of the metestrous rat (43%) (Peluso and Downey, 1982). Thus at any one point in time, more cells are in the S-phase of the cell cycle in the rat follicle than in the mouse follicle. This could account for the shorter apparent cell cycle length of rat ovarian follicles.

This study also demonstrates that FSH inhibits the growth of mid-sized antral follicles *in vitro*. The growth of mid-sized antral follicles can not be supported by FSH alone (Lostroh and Johnson, 1966), but requires additional LH stimulation (Bogovich *et al.*, 1981). In the presence of FSH, the ovary responds to LH differently depending on the mode of administration even though the total amount of LH exposure is the same. FSH overrides the stimulatory action of tonic LH thereby blocking follicular growth. FSH also inhibits the stimulatory effects of LH pulses with amplitudes of 40 ng/ml on mid-sized antral follicular growth. It is not until LH is delivered in pulses with 50 ng/ml amplitudes that LH can override the inhibitory effects of FSH. This observation suggests that FSH plays an important role in modulating the stimulatory actions of LH on growing mid-sized antral follicles.

Follicular distribution in ovaries treated with LH pulses of 50 ng/ml in amplitude emulate the follicular growth pattern seen in the *in vivo* controls. Thus, treatment with pulsatile LH (50 ng/ml in amplitude) most closely simulates the *in vivo* controls. The difference in cell cycle time between *in vivo* controls (5.8 h) and pulsatile

LH treatment *in vitro* (50 ng/ml in amplitude) (11.9 h) may be due to the lack of prolactin in the culture media. Prolactin stimulates progesterone production (Smith *et al.*, 1980; Veldhuis *et al.*, 1981). Progesterone in turn potentiates the growth of mid-sized antral follicles (Kent, 1973; Peluso *et al.*, 1980b; Richards and Bogovich, 1982). Without prolactin to stimulate progesterone production and progesterone to act synergistically with LH to stimulate granulosa cell divisions, the apparent cell cycle length is longer in the pulsed LH (50 ng/ml in amplitude) *in vitro* treatment group.

LH pulses of 50 ng/ml in amplitude but not 40 ng/ml can override the inhibitory effects of FSH. This result shows that the ovary can respond to a difference in amplitude as small as 10 ng/ml. It may not only be the amplitude of the LH pulse which is important in determining follicular response to LH, but also the rate of change of the LH concentration. The inability of a 40 ng/ml pulse to override the inhibitory effects of FSH on the growth of mid-sized antral follicles may not only be due to an insufficient amplitude, but also an inadequate rate of change of LH concentration. It is proposed that the faster increase in LH levels associated with the 50 ng/ml LH pulse is necessary to counteract the inhibitory effects of FSH on mid-sized antral follicular growth. This possibility is currently under study.

Table 1

Concentrations of LH in the concentrating and diluting media delivered to the culture chamber to induce LH pulses.

	Initial Concentration in Culture Chamber	Concentrating Media	Diluting Media
LH pulse (40ng)	20	44.47	15.53
LH pulse (50ng)	10	58.94	1.06

All concentrations of LH expressed as ng LH-RP-1/ml.

Table 2

The average granulosa cell volume and average cell density of the different treatment groups.

TREATMENT	X GRANULOSA CELL VOLUME* (um^3)	X CELL DENSITY* ($cells/um^2$)
IN VIVO		
1030h	125.5 ± 8.0	8.3 ± 0.37
1330h	119.9 ± 6.5	7.7 ± 0.22
IN VITRO		
No Gonadotropins	93.9 ± 5.1	9.5 ± 0.67
Tonic FSH	104.2 ± 8.2	8.5 ± 0.44
Tonic LH	93.3 ± 6.5	9.6 ± 0.74
FSH + Tonic LH	108.7 ± 5.3	8.9 ± 0.62
FSH + Pulsed LH (20-40-20 ng/ml)	112.5 ± 9.2	9.9 ± 0.40
FSH + Pulsed LH (10-50-10 ng/ml)	100.5 ± 4.5	9.6 ± 0.50

* MEAN ± SEM.

Figure 1. Administration of Luteinizing Hormone (LH) during the 3h culture period. Ovaries were exposed to LH in either tonic (30 ng LH-RP-1/ml) or pulsatile modes. The LH pulses had amplitudes of either 40 ng/ml (-----) or 50 ng/ml (- - -). Regardless of treatment, each ovary was exposed to the same amount of LH (3060 ng/3h).

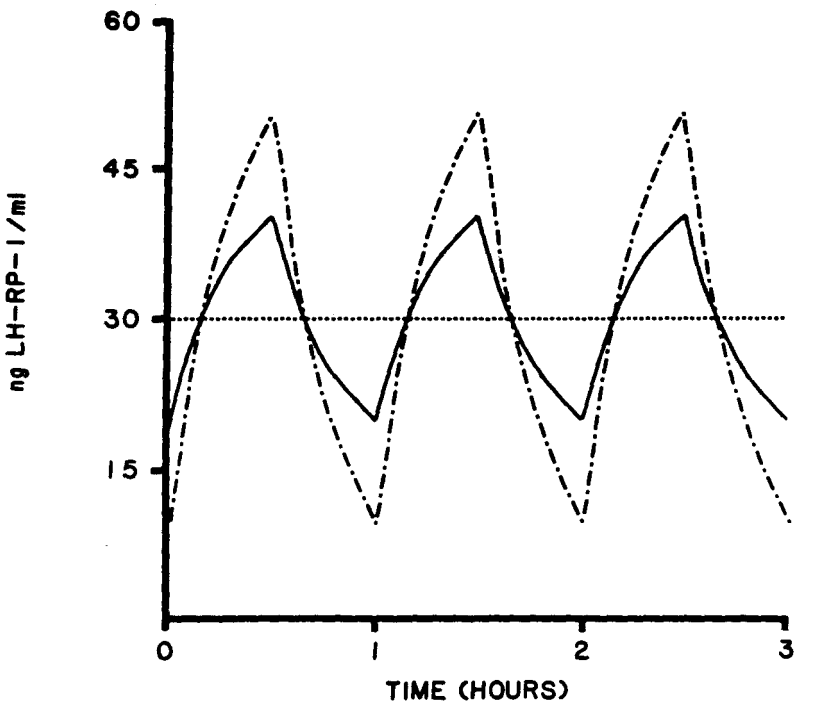

60
45
30
15
ng LH-RP-1/ml
0
1
2
3
TIME (HOURS)

Figure 2. Description of the culture system. Media within each holding flask was gassed with 5% CO_2 in O_2 and delivered to the culture chamber (a 20 ml syringe) at a rate of 34 ml/h by a peristaltic pump. Gas was allowed to enter the holding flask by opening an in-line solenoid valve (ITT-General Controls, Glendale, Ca.; Model S301). The solenoid valve was controlled by a Chrontrol microprocessor controller (Lindberg Enterprises, San Diego, Ca.). The culture chamber was housed in a 37 °C incubator. The system used to deliver tonic hormonal stimuli is depicted in Figure A; whereas the apparatus shown in Figure B was used to deliver pulses of hormone.

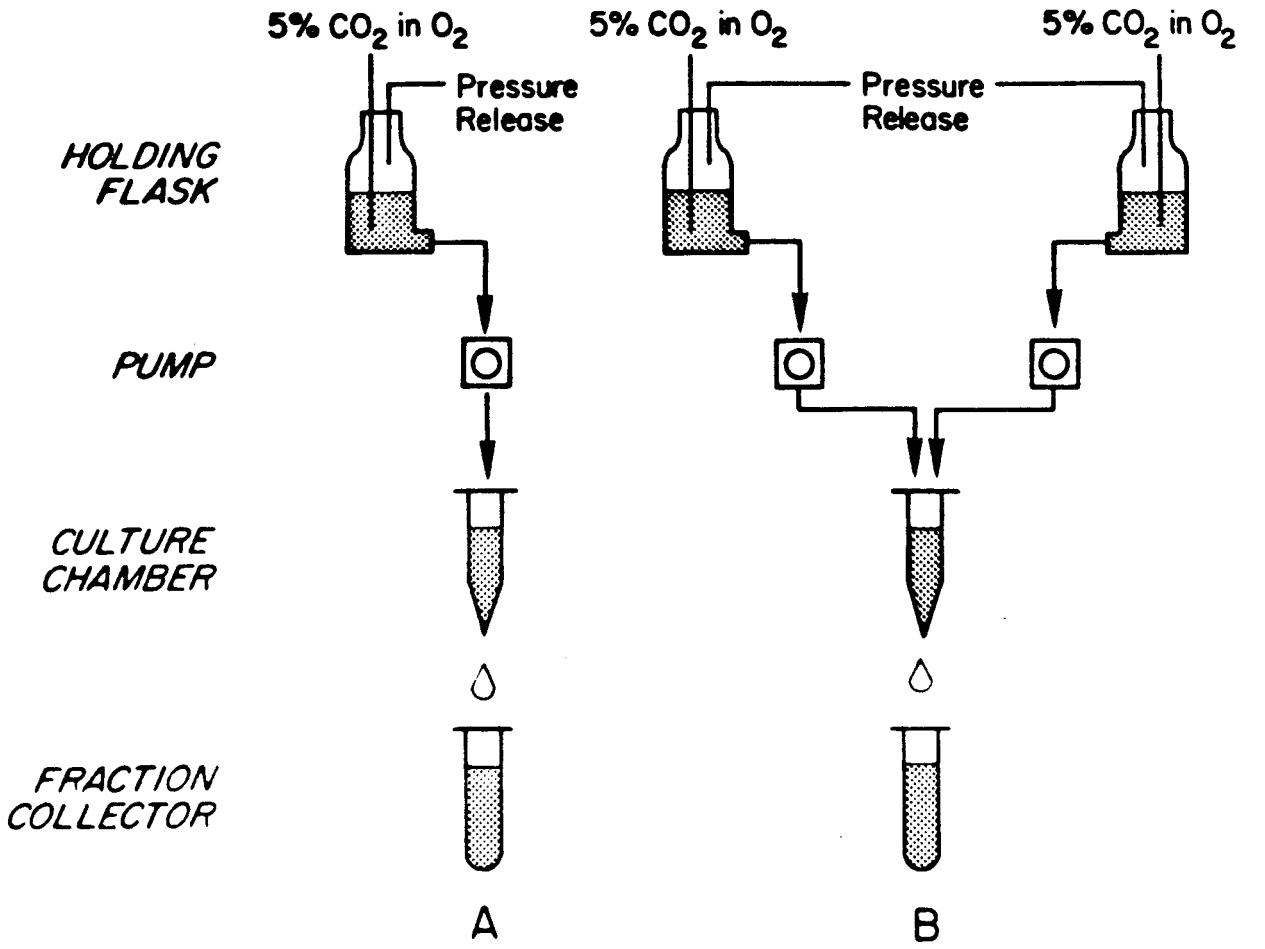

5% CO_2 in O_2
Pressure Release
HOLDING FLASK
PUMP
CULTURE CHAMBER
FRACTION COLLECTOR
A
5% CO_2 in O_2
5% CO_2 in O_2
Pressure Release
B

Figure 3. Follicular distribution within rat ovaries collected at 1030h (upper panel) and 1330h on metestrus (lower panel). Follicles are represented as either non-atretic (*) or atretic (o). Significant differences ($p<0.05$) in the total number of follicles (T. Foll.) and % non-atretic follicles (% Normal) are represented by arrows; no differences are indicated by dashes. One ovary from each of two metestrous rats was examined for the upper panel and three ovaries from two metestrous rats were examined for the bottom panel.

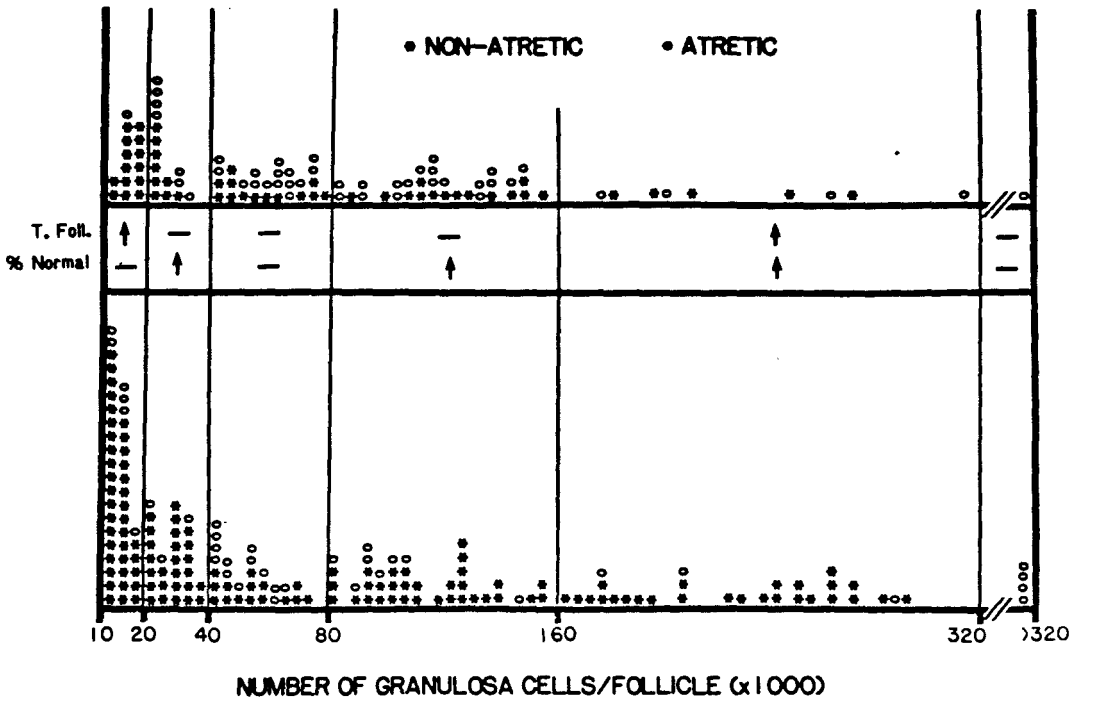

• NON-ATRETIC
• ATRETIC
T. Foll.
% Normal
10
20
40
80
160
320
320
NUMBER OF GRANULOSA CELLS/FOLLICLE (x1000)

Figure 4. Effect of tonic FSH (200 ng FSH-RP-1/ml) on follicular development *in vitro* (lower panel). In Figures 4 to 8, the upper panel represents follicular development after a 3h culture period without hormonal stimuli. Follicles are represented as either non-atretic (*) or atretic (o). Significant differences ($p < 0.05$) in the total number of follicles (T. Foll.) and % non-atretic follicles (% Normal) of the non-hormonal control and various treatment groups are indicated by arrows. A dash indicates no difference. In Figures 4 to 8, three ovaries from two metestrous rats were examined for each treatment unless otherwise specified.

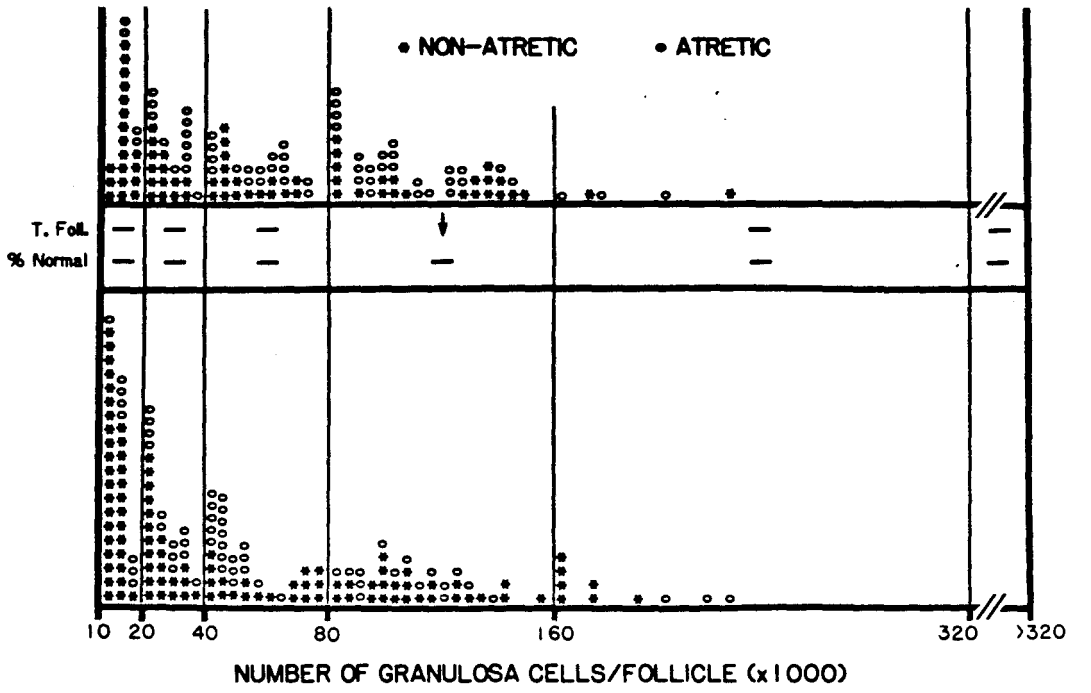

• NON-ATRETIC
• ATRETIC
T. Foll.
% Normal
10
20
40
80
160
320
>320
NUMBER OF GRANULOSA CELLS/FOLLICLE (x1000)

Figure 5. Effect of tonic LH (30 ng LH-RP-1/ml) on follicular development *in vitro* (lower panel).

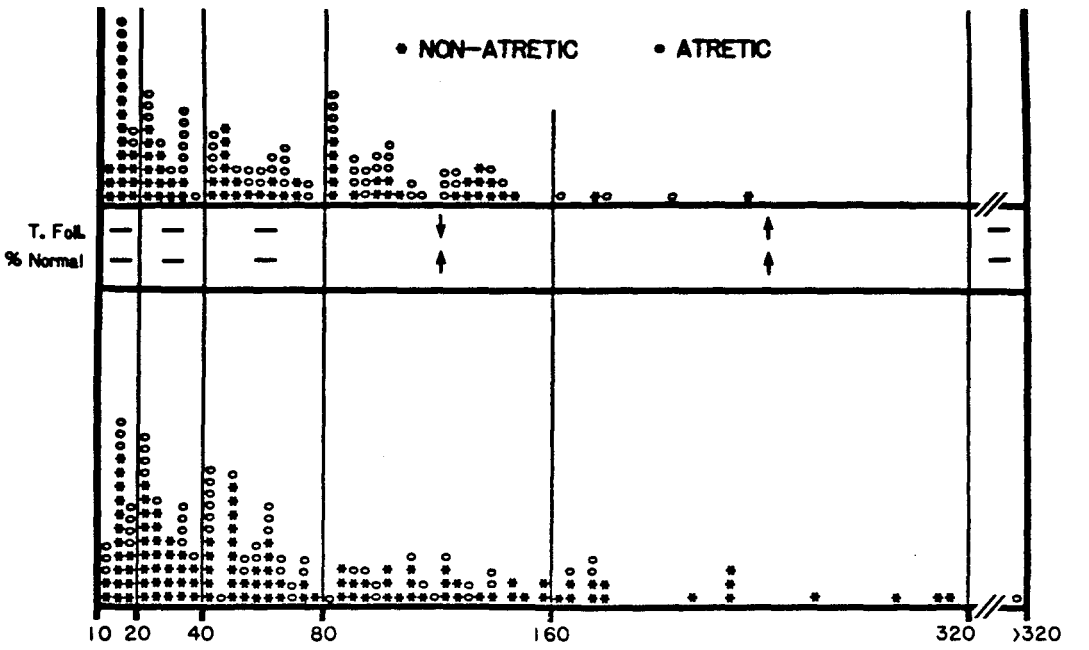

• NON-ATRETIC
• ATRETIC
T. Foll.
% Normal
10 20 40 80 160 320 >320
NUMBER OF GRANULOSA CELLS/FOLLICLE (x1000)

Figure 6. Effect of tonic FSH and tonic LH on follicular development *in vitro* (lower panel).

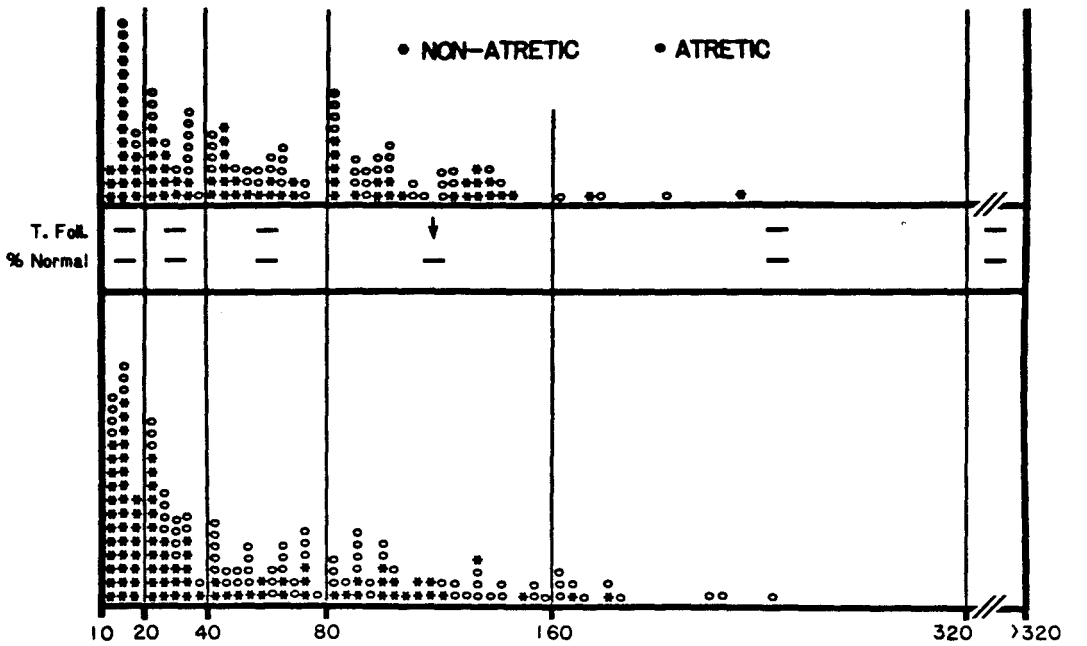

• NON-ATRETIC
• ATRETIC
T. Foll.
% Normal
10
20
40
80
160
320
>320
NUMBER OF GRANULOSA CELLS/FOLLICLE (x1000)

Figure 7. Effect of tonic FSH and pulsatile LH (20ng-40ng-20ng) on follicular development *in vitro* (lower panel). Two ovaries were examined for the hormone treated group.

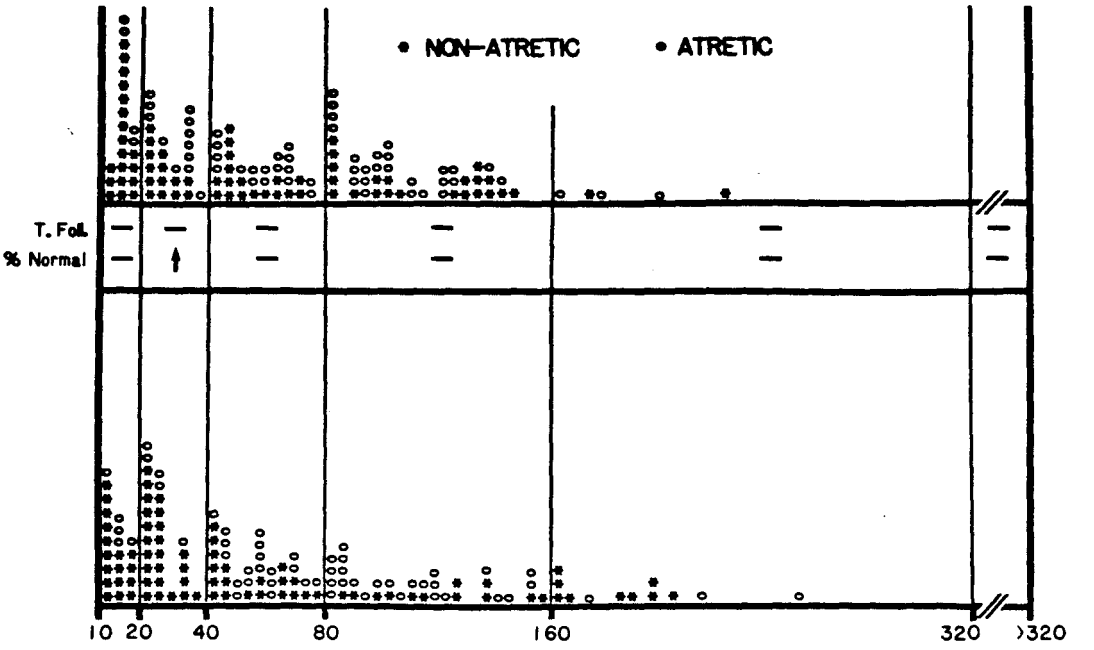

• NON-ATRETIC
• ATRETIC
T. Foll.
% Normal
10 20 40 80 160 320 >320
NUMBER OF GRANULOSA CELLS/FOLLICLE (x1000)

Figure 8. Effect of tonic FSH and pulsatile LH (10ng-50ng-10ng) on follicular development *in vitro* (lower panel).

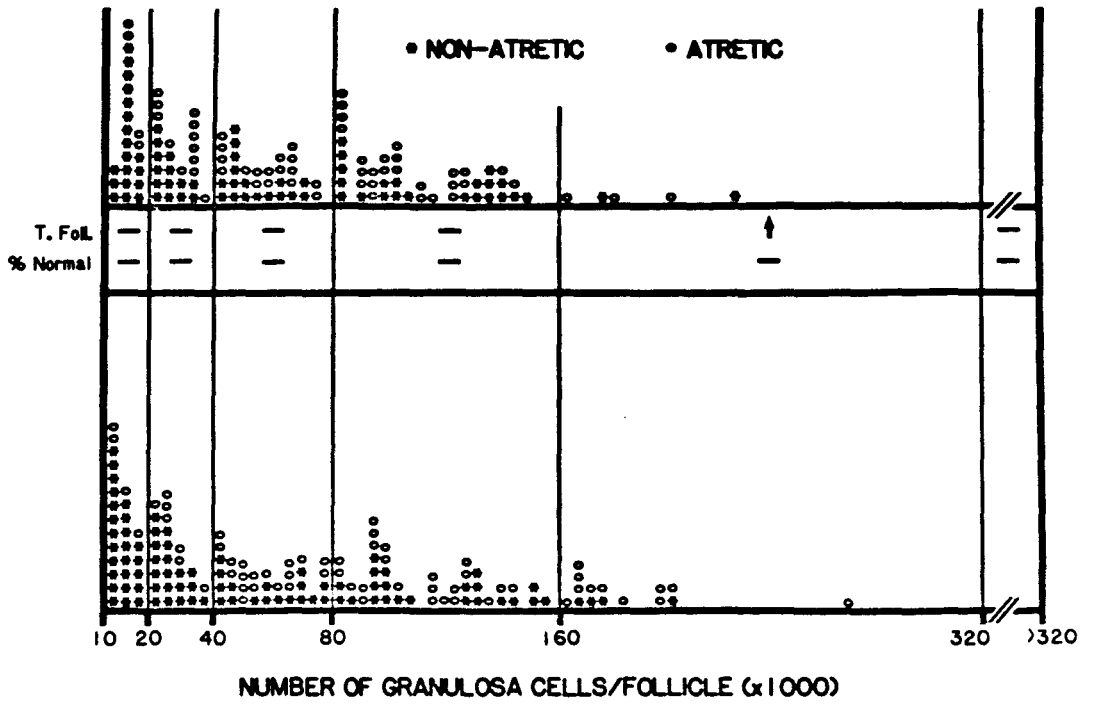

• NON-ATRETIC
• ATRETIC
T. Foll.
% Normal
10
20
40
80
160
320
>320
NUMBER OF GRANULOSA CELLS/FOLLICLE (x1000)

Figure 9. The increase () and % increase () in the number of cells per follicle in response to various treatments either during the 3h in vivo or the 3h culture period. The in vivo groups represent the pairing of follicles from ovaries autopsied at 1030h and 1330h. The effects of tonic LH, and FSH and pulsatile LH (amplitude = 50 ng/ml) on ovaries are also shown. Values shown are the mean ± SEM.

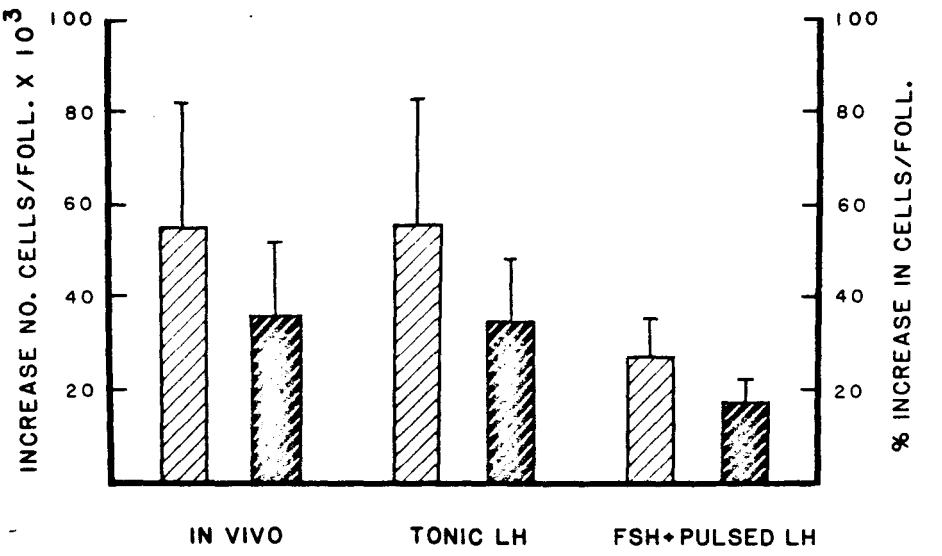
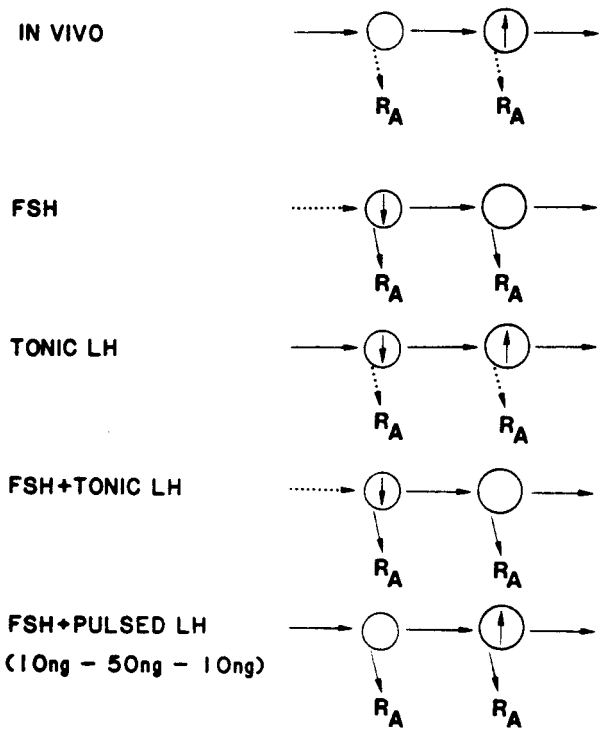

INCREASE NO. CELLS/FOLL. X 10^3
100
80
60
40
20
% INCREASE IN CELLS/FOLL.
100
80
60
40
20
IN VIVO
TONIC LH
FSH+PULSED LH

Figure 10. Hormonal control of follicular growth and atresia. The circles on the left represent the population of follicles with 80-159 x 10^3 cells, whereas the circles on the right represent the population of follicles with 160-320 x 10^3 cells. (R_A) indicates the rate of atresia; vertical arrows represent an increase (↑) or decrease (↓) in the particular population of follicles; (- - >) indicates a decrease in follicular growth and/or atresia.


IN VIVO
FSH
TONIC LH
FSH+TONIC LH
FSH+PULSED LH
(10ng - 50ng - 10ng)
R_A
R_A

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Approval Sheet

The thesis submitted by Shirley Jane Luttmer has been read and approved by the following committee:

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The thesis is therefore accepted in partial fulfullment of the requirements for the degree of Master of Science.

4-20-83
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